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1 **THE COMPLETE PLASMID SEQUENCES OF *SALMONELLA ENTERICA***  
2 **SEROVAR TYPHIMURIUM U288**

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# Abstract

*Salmonella enterica* Serovar Typhimurium U288 is an emerging pathogen of pigs. The strain contains three plasmids of diverse origin that encode traits that are of concern for food security and safety, these include antibiotic resistant determinants, an array of functions that can modify cell physiology and permit genetic mobility. At 148,711 bp, pSTU288-1 appears to be a hybrid plasmid containing a conglomerate of genes found in pSLT of *S. Typhimurium* LT2, coupled with a mosaic of horizontally-acquired elements. Class I integron containing gene cassettes conferring resistance against clinically important antibiotics and compounds are present in pSTU288-1. A curious feature of the plasmid involves the deletion of two genes encoded in the *Salmonella* plasmid virulence operon (*spvR* and *spvA*) following the insertion of a *tnpA* IS26-like element coupled to a *bla*<sub>TEM</sub> gene. The *spv* operon is considered to be a major plasmid-encoded *Salmonella* virulence factor that is essential for the intracellular lifecycle. The loss of the positive regulator SpvR may impact on the pathogenesis of *S. Typhimurium* U288. A second 11,067 bp plasmid designated pSTU288-2 contains further antibiotic resistance determinants, as well as replication and mobilization genes. Finally, a small 4,675 bp plasmid pSTU288-3 was identified containing mobilization genes and a *pleD*-like G-G-D/E-E-F conserved domain protein that modulate intracellular levels of cyclic di-GMP, and are associated with motile to sessile transitions in growth.

**Keywords:** *Salmonella* Typhimurium U288, hybrid virulence plasmid, antibiotic resistance plasmid, class I integron, transposable element, *spv* operon, G-G-D/E-E-F domain.

## 34 1. Introduction

35 The Gram negative bacterial genus *Salmonella* (part of the family *Enterobacteriaceae*) is  
 36 composed of two distinct facultative anaerobic species: - *S. enterica* and *S. bongori* (Fluit,  
 37 2005). The six subspecies that define *S. enterica* (*enterica* [I], *salamae* [II]), *arizonae* [IIIA],  
 38 *diarizonae* [IIIB], *houtenae* [IV], and *indica* [VI]) constitute in excess of 2,500 recognized  
 39 serovars. *S. bongori* previously composed group V prior to recognition as an independent  
 40 lineage (Fluit, 2005; Fookes et al., 2011; Lan et al., 2009). Whilst *S. bongori* is most often  
 41 associated with infections of ectothermic animals such as lizards, there are limited reports of  
 42 human infection (Fookes et al., 2011). In contrast, many of the serovars that form the group  
 43 *S. enterica* are considered to be significant human and animal pathogens (Fookes et al.,  
 44 2011), with *S. enterica* implicated as the causative agent in >99% of identified salmonellosis  
 45 incidents in humans (Jacobsen et al., 2011). The majority of *S. enterica* that cause disease  
 46 can be further grouped in terms of their host range, for instance, host-restricted serovars *S.*  
 47 *Typhi* and *S. Paratyphi A* are limited to causing typhoid fever in humans (Baker and Dougan,  
 48 2007; Holt et al., 2008; Parkhill et al., 2001) whilst the causative agents of fowl typhoid - *S.*  
 49 *Gallinarum* and pig paratyphoid - *S. Choleraesuis* are similarly host-restricted (Thomson et  
 50 al., 2008). However, such serovars retain the ability to infect other animals, often with severe  
 51 consequences in terms of disease-outcomes (Betancor et al., 2012; Ye et al., 2011). The  
 52 broad host range serovars, notable examples include *S. Enteritidis* PT4 (Thomson et al.,  
 53 2008) and *S. Typhimurium* DT104, are capable of causing gastrointestinal infections in a  
 54 wide range of hosts with *S. Typhimurium* DT104 capable of infecting both humans and food-  
 55 producing animals such as cattle, sheep, and pigs (Cooke et al., 2008; Gebreyes and Altier,  
 56 2002; Mulvey et al., 2006).

Over the last decade *S. Typhimurium* U288 has emerged as a significant pathogen of pigs in the UK and is now the serovar most often identified through surveillance programmes by the UK Veterinary Laboratories Agency (Mueller-Doblies et al., 2013). It has previously been reported that many *S. Typhimurium* U288 isolates of porcine origin possess a transmissible 150 kilobase (kb) plasmid, often found in tandem with smaller 14 and 3 kb plasmids. Analysis of the resistance phenotypes and associated genotypes of each *S. Typhimurium* U288 revealed the presence of determinants that provide protection against an array of antibiotics of clinical importance (Anjum et al., 2011) with the most commonly displayed antibiotic resistance profile being AmCSSuTTm (VLA, 2012). In order to further our understanding of *S. Typhimurium* U288, a whole genome sequencing approach was employed (Hooton et al., 2013). Following assembly of the sequence data obtained, three separate plasmids were constructed and analyzed and are presented in the course of this work.

## 2. Materials & methods

### 2.1 Plasmid DNA sequencing and characterisation

The complete genome of *S. Typhimurium* U288 was sequenced from genomic DNA using the Roche 454 GS FLX sequencing system (Roche Diagnostics, USA) as described previously (Hooton et al., 2013). Following *de novo* assembly of the genomic DNA sequence reads, a single contig of 4,852,606 bp representing the *S. Typhimurium* U288 chromosome was constructed (Genbank Acc. No. CP003836). Sequence reads that did not map to the chromosome were then independently assembled into three circular plasmid DNAs ready for annotation (148,711 bp pSTU288-1, 11,067 bp pSTU288-2, and 4,675 bp pSTU288-3). Confirmation of the presence of three plasmids in *S. Typhimurium* U288 that correspond to those predicted from the assembled sequence reads was achieved by analysis of plasmid preparations. Briefly, an overnight culture of *S. Typhimurium* U288 was subjected

81 to GenElute™ Plasmid Miniprep extraction as per manufacturer's instructions (Sigma  
 82 Aldrich, UK). Eluate from the miniprep was electrophoresed in a 0.7% agarose ethidium  
 83 bromide-stained gel and visualized using a Biorad Image Capture (Biorad, USA). Plasmid  
 84 preparations were used to transform competent *E. coli* DH5 $\alpha$  that enabled the efficient  
 85 recovery of the antibiotic resistance plasmid pSTU228-2 by selection on LB-agar plates  
 86 containing either streptomycin (10  $\mu$ g/mL) or tetracycline (10  $\mu$ g/mL) or chloramphenicol  
 87 (30  $\mu$ g/mL) or neomycin (10  $\mu$ g/mL).

## 88 2.2 DNA sequence analysis and annotation

89 Several different methods were employed in order to annotate the three plasmids including  
 90 NCBI PGAAP (<http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html>). For pSTU288-  
 91 1, xBASE2 automatic annotation pipeline was used with pSLT of *S. Typhimurium* LT2 as the  
 92 reference sequence (Chaudhuri et al., 2008). Regions of the pSTU288-1 genetic map which  
 93 were insufficiently annotated through PGAAP and xBASE2 were manually curated using a  
 94 combination of BLASTN searching (Altschul et al., 1990) and the Artemis sequence viewing  
 95 tool (Rutherford et al., 2000). Due to the small sizes of pSTU288-2 and pSTU288-3 each  
 96 plasmid was manually curated as described above. When necessary, translated amino acid  
 97 sequences from open reading frames (ORFs) identified on each plasmid were subjected to  
 98 protein domain searches using the Pfam database (Finn et al., 2010). All pairwise alignments  
 99 of protein and DNA sequences were performed using ClustalW2 with default settings (Larkin  
 100 et al., 2007). The Integrall database was accessed to assign integron families (Moura et al.,  
 101 2009). The fully annotated plasmids were deposited in Genbank under the following  
 102 accession numbers - pSTU288-1 (CP004058), pSTU228-2 (CP004059), and pSTU288-3  
 103 (CP004060).

## 104 2.3 Conjugation

Mating experiments were carried out by mixing exponential growth phase broth cultures of donor *S. Typhimurium* U288 and recipient *E. coli* DH5 $\alpha$  at a ratio of approximately 1:3 on LB-agar plates and incubating for six hours at 37 °C. The mating mixture was resuspended in LB medium and plated as serial dilutions on LB-agar plates containing nalidixic acid (50  $\mu$ g/mL) to counter-select *S. Typhimurium* U288 and streptomycin (10  $\mu$ g/mL) for plasmids replicating in *E. coli* DH5 $\alpha$ . LB-agar plates containing either ampicillin (25  $\mu$ g/mL) or tetracycline (10  $\mu$ g/mL) or trimethoprim (25  $\mu$ g/mL) were used to discriminate the respective carriage of plasmids pSTU288-1 and pSTU228-2. Plasmid transfer frequencies are expressed as number of transconjugants per donor cell. Donor cells were enumerated by plating serial dilutions onto xylose lysine desoxycholate agar (XLD; Oxoid, Basingstoke UK).

### 3 Results

#### 3.1 pSTU288-1 large virulence and antibiotic resistance plasmid

The largest of the plasmids identified following whole genome sequencing of *S. Typhimurium* U288 was the 148,711 bp large virulence and antibiotic resistance plasmid pSTU288-1 (Figure 1). The GC content of pSTU288-1 was observed to be 52.95% which is marginally above the 52.18% GC content determined for the *S. Typhimurium* U288 chromosome, but similar to other large *Salmonella* virulence plasmid sequences deposited in the database. BLASTN analysis of the nucleotide sequence of pSTU288-1 reveals that its closest relative is the 93,939 bp pSLT plasmid (GenBank Acc. No. AE006471) of *S. Typhimurium* LT2 (McClelland et al., 2001). It should be noted that many other *S. Typhimurium* plasmid sequences also align over various regions of the pSTU288-1 sequence. However, it is apparent that pSTU288-1 is a unique hybrid plasmid containing highly conserved regions comparable with pSLT, accompanied by multiple regions that have been

129 horizontally-acquired over time. The positions and sequence-related origins are indicated in  
 130 the inner track of Figure 1. Based on nucleotide sequence alignments between pSTU288-1  
 131 and pSLT approximately 85 kb of sequence is highly conserved between the two plasmids.  
 132 The conserved regions are dispersed throughout the plasmid with the largest continuous  
 133 region of 56,732 bp showing 99% identity.

134 Numbering from a Transposase 31-like element insertion within the circular sequence,  
 135 BLASTN analysis indicates the first 20,079 nucleotides of pSTU288-1 have 99% identity to a  
 136 region of pSL476\_91, a 91kbp plasmid from *S. Heidelberg* str. SL476 (GenBank Acc. No.  
 137 CP001118). This sequence is notably duplicated in pSTU288-1 as it reoccurs between ~68  
 138 kb and 88 kb on the circular sequence of the plasmid. The genes found within the duplicated  
 139 regions show a high degree of synteny when compared to each other but are clearly  
 140 distinguishable by numerous insertions and deletions, accompanied by complete breakdown  
 141 of homology in intergenic non-coding regions. Analysis of the genes found within the  
 142 duplicated regions show that both are flanked by a Transposase 31-like element (pSTU288-  
 143 1\_0001 and pSTU288-1\_0122). The amino acid sequences deduced for the transposases  
 144 show a high degree of conservation over the first 200 amino acids that are recognizable as  
 145 transposase 31 family protein domains (PF04754 PD(D/E)XK nuclease superfamily -  
 146 CL0236), which encompasses a diverse range of nucleases and Holliday junction resolvases  
 147 (Feder and Bujnicki, 2005). The presence of these transposases at the end of each duplicate  
 148 sequence suggests a possible mechanism for transposition. Two further genes of known  
 149 function present in the pSTU288-1 duplicated regions that are absent from the corresponding  
 150 pSLT region are *ardA* (pSTU288-1\_0005) and *ardB* (pSTU288-1\_0015), which encode  
 151 proteins belonging to the respective ArdA (PF07275) and ArdB (PF03230) anti-restriction  
 152 families. The *ardA* and *ardB* genes show a high degree of nucleotide sequence conservation



with homologous genes identified in plasmids isolated from several *Salmonella* serovars, as well as a large number of *E. coli* sequences.

Nucleotide sequence conservation between pSTU288-1 and pSLT begins at nucleotide 20,720 with a 12.5 kb region (12,445/12,500 bases) that contains several genes including a replication initiation protein (*repA2* - pSTU288-1\_0032), a conserved OB-fold protein (*ygiW* - pSTU288-1\_0033), as well as the virulence-associated *pef* operon (pSTU288-1\_0034 to pSTU288-1\_0042). Immediately downstream of pSTU288-1\_0045 a complete breakdown in conservation with pSLT is observed due to the presence of a TnIS66 transposable element (pSTU288-1\_0043 to 0045). There is an identical TnIS66 element present at nucleotide positions 145,640 to 148,255 of pSTU288-1, as well as a further example integrated in the *S. Typhimurium* U288 chromosome (STU288\_09455, STU288\_09460, and STU288\_09465) that suggests the element continues to be active. Synteny between pSTU288-1 and pSLT is restored after the transposable element resulting in 99% conservation over 64 kb of sequence (bases 35,917-99,920) that features genes involved in virulence, plasmid replication and conjugal transfer.

The region spanning nucleotides 121,821-132,269 in pSTU288-1 is conserved with pSLT, which contains several recognizable genes including the *ccdA* and *ccdB* genes associated with plasmid maintenance, a resolvase encoded by *rsdB*, an intimin-like protein encoded by *sinH* and a truncated *spv* (*Salmonella* plasmid virulence) operon. The pSTU288-1 *spv* operon has the configuration *spvBCDE* that departs from the *spvRABCDE* configuration observed elsewhere (Gulig et al., 1993). Analysis of the sequence environment indicates that the *spvR* and *spvA* genes have been deleted in pSTU288-1 following the insertion of a  $\beta$ -lactamase gene (*bla*<sub>TEM</sub>) coupled to a transposon. Figure 2 shows a comparison between the truncated *spv* region of pSTU288-1 and the intact region found on pSLT. The transposable element responsible for the deletion of *spvRA* encodes genes *tnpA* (pSTU288-1\_0175), *tnpR*

178 (pSTU288-1\_0176) and *bla*<sub>TEM</sub> (pSTU288-1\_0177). TnpA is a 238 amino acid protein that  
 179 contains a definable DDE\_Tnp\_IS240 protein domain (PF13610) of the RNase H-like  
 180 Superfamily (CL0219) spanning residues 77-217. BLASTP analysis shows that the TnpA  
 181 protein is conserved amongst many pathogenic bacteria but notably has 100% identity with  
 182 the TnpA IS26 elements found in plasmids of *S. Choleraesuis* and *S. Typhi* CT18. The *tnpR*  
 183 gene encodes a short 53 amino acid protein (molecular weight 5.9 kDa.) that contains a  
 184 HTH\_7 (helix-turn-helix) resolvase domain (PF02796 - HTH clan CL0123), which is highly  
 185 conserved with several dozen *Klebsiella pneumoniae* and *E. coli* 0104:H4 TnpR sequences in  
 186 public databases. The *bla*<sub>TEM</sub> gene encodes a 286 amino acid protein that belongs to the  $\beta$ -  
 187 lactamase 2 enzyme family (PF13354), part of the Serine  $\beta$ -lactamase-like Superfamily  
 188 (CL0013).

189 The sequences of the plasmid replication gene *repA* (pSTU288-1\_0054) and its regulatory  
 190 partner *repB* (pSTU288-1\_0055) indicate that pSTU288-1 is an IncFII incompatibility group  
 191 plasmid. The 293 amino acid RepA protein contains a definable IncFII RepA protein family  
 192 domain (PF02387) from residues 10-276. The 88 amino acid RepB protein is a member of  
 193 the RepB RCR regulatory protein family (PF10723) that regulate plasmid rolling circle  
 194 replication (Khan, 1997). The genes encoding all the necessary functions for conjugal  
 195 transfer (the *tra* genes) are located between nucleotides 42,034-77,017 (pSTU288-1\_0056 to  
 196 pSTU288-1\_0096) on the genetic map.

### 197 **3.2 The class I integron of pSTU288-1**

198 A class I integron (In640) is located between nucleotides 99,672 and 114,690 within  
 199 pSTU288-1 flanked by two large transposable elements. The 3'-CS (conserved sequence) of  
 200 the class I integron is flanked by an IS26 element linked to genes encoding FabG (pSTU288-  
 201 1\_0134), SulIII (pSTU288-1\_0137) and several conserved hypothetical proteins. The *tnpA*-

202 *IS26* transposase gene (pSTU288-3\_0131) encodes a 238 amino acid protein containing a  
 203 DDE\_Tnp\_IS240 domain (PF13610) of the RNaseH-like superfamily (CL0219).  
 204 Immediately downstream of *sulIII* is a *tnp\_mut* gene (pSTU288-1\_0138) that appears to have  
 205 been inserted into the 3'-CS creating an atypical class I integron structure. Typical class I  
 206 integrons are found to contain a *qac* gene fused to a *sulI* gene and it is this partnership that  
 207 defines the 3'-CS region (Chuanchuen et al., 2010; Dawes et al., 2010). The atypical  
 208 configuration of the 3'-CS in pSTU288-1 consists of a *qacEAI/tnp\_mut/sulIII* combination.  
 209 The *intI1* gene (pSTU288-1\_0145) defines the 5'-CS region of the class I integron of  
 210 pSTU288-1. The gene encodes a 337 amino acid protein containing two phage integrase  
 211 domains that function in a similar manner to the site-specific tyrosine recombinase XerD.  
 212 The first protein domain spans residues 16-100 and belongs to the Phage Integrase N2 N-  
 213 terminal SAM-like domain family (PF13495 –  $\lambda$ -integrase N-terminal domain CL0469).  
 214 Amino acid residues 115-319 encompass a second Phage integrase domain (PF00589) that is  
 215 part of the DNA Breaking/Rejoining enzyme superfamily (CL0382). The *intI1* gene of  
 216 pSTU288-1 is situated adjacent to three genes encoding a Tn21 element – *tnpM* (pSTU288-  
 217 1\_0146), *tnpR* (pSTU288-1\_0147), and *tnpA* (pSTU288-1\_0148). Encoded within the  
 218 pSTU288-1 class I integron are a number of gene cassettes that confer resistance to several  
 219 antibiotic classes detailed in Table 1.

220 Adjacent to the Tn21 element (pSTU288-1\_0149 - pSTU288-1\_0158) is a 7,097 bp region  
 221 that aligns with plasmids pSal8934a (GenBank Acc. No. JF274933) of *S. Typhimurium*  
 222 (100% identity) and pLM (GenBank Acc. No. JQ901381) of *E. coli* str. EC25 (99% identity).  
 223 The final 16 kb of pSTU288-1 appears to be a hybrid of plasmid sequences of diverse origin  
 224 but includes three genes associated with plasmid conjugal transfer – *trbA* (pSTU288-  
 225 1\_0181), *trbB* (pSTU288-1\_0182), and *trbC* (pSTU288-1\_0183), and the plasmid  
 226 mobilization genes *nikA* (pSTU288-1\_0185) and *nikB* (pSTU288-1\_0186).

227

### 228 3.3 Plasmid conjugal transfer

229 Plasmid pSTU288-1 could be efficiently transferred by conjugation from *S. Typhimurium*  
230 U288 to *E. coli* DH5 $\alpha$  at a frequency of  $2 (\pm 0.8) \times 10^{-4}$  transconjugants per donor cell by  
231 selection for streptomycin resistance. The antibiotic resistance profiles of the transconjugants  
232 confirmed the presence of functional resistance determinants against streptomycin,  
233 ampicillin, chloramphenicol and trimethoprim for pSTU288-1. The presence of tetracycline  
234 resistance in >90% of the transconjugants implied the efficient co-transfer of pSTU288-2.  
235 Plasmid DNAs were recovered from tetracycline resistant transconjugants and used to  
236 transform *E. coli* DH5 $\alpha$  with selection for tetracycline resistance. Plasmid DNAs could be  
237 recovered from the *E. coli* DH5 $\alpha$  transformants that corresponded with the expected size of  
238 pSTU288-2 upon agarose gel electrophoresis. The transformants exhibited resistance to the  
239 antibiotics streptomycin, tetracycline, chloramphenicol and neomycin that is consistent with  
240 the profile expected of pSTU288-2.

241

### 242 3.4 pSTU288-2 antibiotic resistance plasmid

243 Plasmid pSTU288-2 is 11,067 bp in size that is notable by its GC content of 61.76%, which  
244 is well above 52.18% observed for the *S. Typhimurium* U288 chromosome. The plasmid  
245 encodes a complement of genes that confer resistance to antibiotics of both medical and  
246 veterinary importance (Table 1). Plasmid preparations of *S. Typhimurium* U288 were used to  
247 transform *E. coli* DH5 $\alpha$  with selection for either streptomycin or tetracycline resistance.  
248 Plasmid DNAs of pSTU288-2 could be recovered from the *E. coli* DH5 $\alpha$  transformants based  
249 on DNA sequencing data. The antibiotic profiles conferred were similar to those noted above

for pSTU288-2 (streptomycin, tetracycline, chloramphenicol and neomycin), and confirm the potential of the plasmid to transfer functional antibiotic resistance determinants. The plasmid shares 100% identity over 6,435 bases with pTY474p3 of *S. Typhimurium* 4/74 (GenBank Acc. No. CP002490). The DNA sequence of pSTU288-2 is numbered from recognizable mobilization and replication open reading frames. Translations of *mobA* (pSTU288-2\_002) and *mobC* (pSTU288-2\_001) are identical with those from a broad host range multicopy IncQ plasmid pRSF1010 (GenBank Acc. No. NC\_001740) isolated from *E. coli* (Scholz et al., 1989). The MobA protein contains a MobA/MobL domain (PF03389 - Rep-like domain CL0169) associated with plasmid transfer. Rep-like domain proteins are known to bind DNA and to contain a conserved central His-X-His motif, which is present in pSTU288-2 MobA as a His-Cys-His motif at residues 120-122. The following three genes on the plasmid are involved in replication of pSTU288-2 - *repF* (partial), *repA*, and *repC*.

### 3.5 pSTU288-3 plasmid

*S. Typhimurium* U288 was also found to possess a small plasmid of 4,675 bp designated pSTU288-3. The GC content of pSTU288-3 (50.99%) is slightly lower than that of the *S. Typhimurium* U288 chromosome. At the nucleotide level pSTU288-3 shares 99% identity with two other small plasmids in the database: pSD4.6 (GenBank Acc. No. JX566768) a small cryptic plasmid carried by an *S. Derby* isolate from pork meat (Bleicher et al., 2013) and pEC278 (GenBank Acc. No. AY589571.1) of *E. coli* 278B. Whilst the similarities between plasmids of equivalent size to pSTU288-3 are restricted to *E. coli* pEC278 and *S. Derby* pSD4.6, it is apparent that segments of the pSTU288-3 nucleotide sequence are present in many plasmid sequences deposited in the GenBank database. Several larger plasmids of approximately 10 kb in length contain the complete pSTU288-3 nucleotide sequence accompanied by an extra 5 kb region that encodes an IS2 transposable element and

274 a gene conferring resistance to quinolones (*qnrS1*). All of the 10 kb quinolone resistance  
 275 plasmids are found in *S. Typhimurium* hosts of various origins: pHLR25 (GenBank Acc. No.  
 276 HE652087) of *S. Typhimurium* HLR25 isolated from a human case of salmonellosis,  
 277 pTPqnrS-1a plasmid (GenBank Acc. No. AM746977) from a multiple drug resistant *S.*  
 278 *Typhimurium* DT193 strain (Kehrenberg et al., 2007), pQnrS1-cp17s (GenBank Acc. No.  
 279 JN393220) of *S. Typhimurium* 484 isolated from frozen eel chunks, and pST728/06-2  
 280 (GenBank Acc. No. EU715253) of an *S. Typhimurium* strain isolated from a human case of  
 281 salmonellosis (Wu et al., 2008).

282 The pSTU288-3 nucleotide sequence was aligned with the start position of *E. coli* pEC278  
 283 prior to opening up at base position 1. Analysis of all potential open reading frames on the  
 284 plasmid reveals a total of five coding sequences. The first gene identified on pSTU288-3 is  
 285 *mobC* (pSTU288-3\_001), which encodes the signature residues (49-96) of the MobC family  
 286 (PF05713) of bacterial mobilization proteins. Immediately downstream of *mobC* is the gene  
 287 encoding the relaxase component of the relaxosome – *mobA* (pSTU288-3\_002). The 499  
 288 amino acid MobA protein contains a relaxase/mobilization domain (PF03432) spanning  
 289 residues 49-247 of the Rep-like domain clan (CL0169). For pSTU288-3 MobA, the  
 290 conserved central motif His-Asp-His can be found at residues 337-339, which is conserved in  
 291 the majority of relaxase/mobilization domain-containing MobA proteins present in the  
 292 database. Located within the *mobA* gene, but +1 in terms of their reading frame, are two co-  
 293 transcribed genes - *mobB* (pSTU288-3\_003) and *mobD* (pSTU288-3\_004). The protein  
 294 products of these two genes form the remaining components of the relaxosome complex:  
 295 MobB 161 amino acid and MobD 71 amino acid. The MobB protein of pSTU288-3 contains  
 296 an MbeB-like conserved N-terminal domain (PF04837) situated between residues 1-52. The  
 297 MobD protein contains an MbeD/MobD-like domain (PF04899) spanning the total length of  
 298 the protein that is also associated with plasmid mobilization and transfer. The final gene

identified in pSTU288-3 designated *pleD*-like (pSTU288-3\_005) encodes a 502 amino acid protein with an estimated molecular weight of 55.4 kDa. The *PleD*-like protein is found to belong to the G-G-D/E-E-F domain family (PF00990) of the nucleotidyl cyclase superfamily (CL0276). BLASTP analysis of the 502 amino acid *PleD*-like protein reveals only one true homologue in the database - the 520 amino acid hypothetical protein pSD4.6\_002 of *S. Derby* plasmid pSD4.6 (496/502 identities - 99%) that also contains a conserved G-G-E-E-F domain motif.

#### 4. Discussion

The three plasmids identified in *S. Typhimurium* U288 are of significant concern when viewed from clinical, veterinary and food security standpoints. *S. Typhimurium* U288 has the potential to acquire yet further antibiotic resistance genes as it appears to have efficiently integrated mobile DNA from many exogenous sources as indicated by the transposable elements containing *bla*<sub>TEM</sub> found in pSTU288-1 and the presence of both plasmid- and chromosomally-integrated TnIS66 elements. *S. Typhimurium* U288 also has the potential to disseminate antibiotic resistance since it encodes the apparatus for conjugal transfer and the plasmids it harbours are mobilizable.

The transposition event that has disrupted the *spv* operon appears a unique occurrence to this *S. Typhimurium* U288 isolate, with no other *S. enterica* *spv*-containing plasmids possessing this configuration to date. This event could alter the course of infection and provide a reason as to why this particular strain has emerged as a successful and persistent pathogen within UK pig production units. The *spv* operon is considered to be a major virulence factor of *Salmonella* by performing a vital role during the infection process (Matsui et al., 2001). Two genes in particular (*spvB* and *spvC*) are recognized as being essential for the bacterium to survive during the intracellular stages of infection, and subsequent systemic dissemination



(Haneda et al., 2012; Mazurkiewicz et al., 2008). The co-ordinated expression of the *spv* genes is dependent on RpoS (stationary phase  $\sigma$  factor) and the LysR family transcriptional regulator SpvR. SpvR is recognized as being a positive regulator of the *spv* operon via transcription of itself and *spvA* (Fabrega and Vila, 2013; Heiskanen et al., 1994), and is expressed in a temporal fashion during infection. *In vitro* induction of *spvR* is observed as cells are entering the stationary phase of growth in liquid culture (Dodd and Aldsworth, 2002), and *in vivo* induction is associated with the transition from the gastrointestinal to intracellular stages of infection (Kappeli et al., 2011). The absence of *spvR* and *spvA* on pSTU288-1 is likely to impact on the coordination of these events. However, the acquisition of the *bla*<sub>TEM</sub> gene is likely to be of significant benefit since it is considered to be a progenitor of the emerging extended-spectrum  $\beta$ -lactamase genes that are currently of global concern (Thomson, 2001). There are many reports in the literature of  $\beta$ -lactamase genes being linked to transposable elements. For example, *S. Choleraesuis* SC-B67 has a *bla*<sub>CMY-2</sub> gene (conferring resistance to ceftriaxone) linked to a *tnpA* element in a similar fashion as that observed for pSTU228-1 (Chiu et al., 2006), as does *S. Braenderup* (Chiou et al., 2009). The selective benefit of carrying the *bla*<sub>TEM</sub> gene would complement the resistance genes already found distributed on the pSTU288-1 and pSTU288-2 plasmids.

The presence of the PleD-like G-G-D/E-E-F domain-containing protein encoded by pSTU288-3 may play a role in allowing *S. Typhimurium* U288 to persist in the environment. Members of this protein family are associated with the synthesis of the intracellular signalling molecule cyclic di-GMP that has been found in the sequences of many bacterial genomes (Ryjenkov et al., 2005). Cyclic di-GMP is a ubiquitous secondary messenger of bacterial signalling and has been associated with many functions in pathogenic bacteria such as control of biofilm formation, cell cycle, cell differentiation and expression of virulence-associated traits (Romling et al., 2013). The PleD-like protein of pSTU288-3 contains a G-G-E-E-F



motif that has the potential to confer a regulatory function that would give *S. Typhimurium* U288 the ability to persist in pig production units within biofilms.

It is apparent that a number of recombination and transposition events in the history of pSTU288-1 have allowed development from an ancestral pSLT-like plasmid to take place. Many of the genes in pSTU288-1 appear to have been acquired from *E. coli* strains, with some of them showing little dispersal throughout *S. enterica*. An interesting example of this in pSTU288-1 is the presence of the gene encoding ArdA (alleviation of restriction of DNA). Members of this protein family are recognized as providing an anti-restriction mechanism against Type I restriction-modification (R-M) systems. ArdA proteins have previously been shown to have the ability to inactivate type I R-M systems in two different ways. ArdA has been observed to have affinity to methyltransferase proteins associated with methylation of DNA, as well as having the ability to bind intact R-M complexes. These two specific actions of ArdA allows unmodified plasmid DNA (lacking the cognate modification pattern of the target cell) to enter a recipient cell during conjugation, protected from restriction and modification by type I R-M systems (Nekrasov et al., 2007; Thomas et al., 2003).

In summary, *S. Typhimurium* U288 has been demonstrated to possess an exceptional complement of plasmid-borne antibiotic resistance and virulence genes, with the prospect that this may be further enhanced in the future via a number of different mechanisms. The presence of pathogenic microorganisms such as *S. Typhimurium* U288 that display resistance to multiple antibiotic classes, especially within food production environments is of great concern. Whilst it appears that this serovar is adapted to pigs, we have demonstrated that *S. Typhimurium* U288 is capable disseminating antibiotic resistance genes to other microorganisms.

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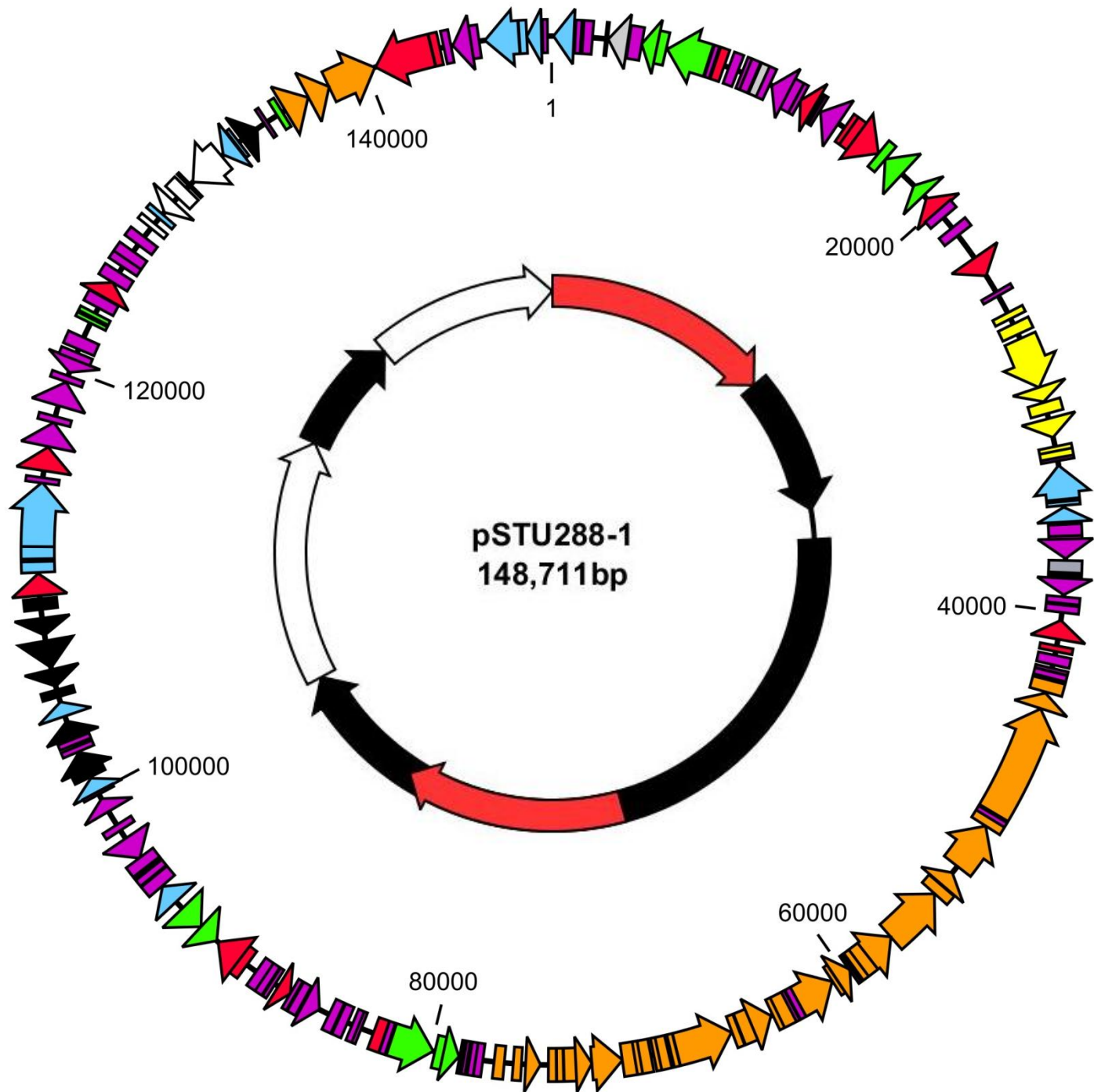
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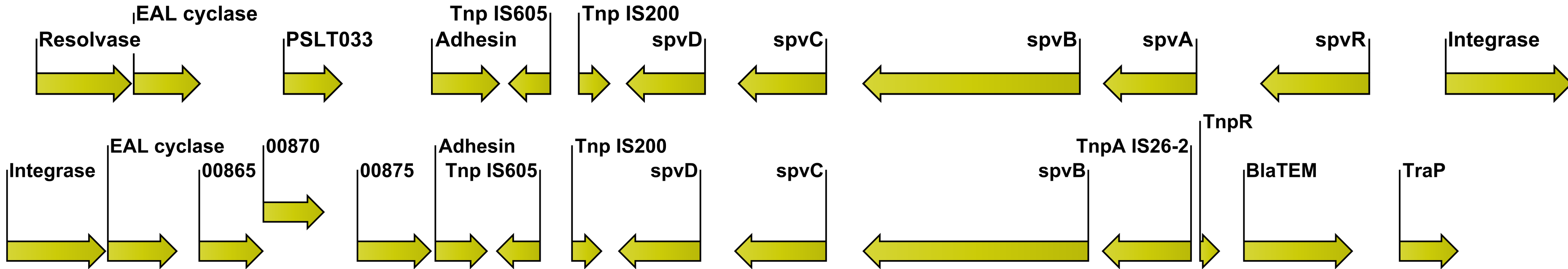
566

567 **Figure 1. A circular representation of the 148,711 bp virulence and antibiotic resistance**  
 568 **plasmid pSTU288-1 of *S. Typhimurium* U288. Inner circle - blue arrows (transposons),**  
 569 **grey (anti-restriction proteins), red (DNA maintenance/methylation/replication proteins),**  
 570 **purple (hypothetical proteins), green (plasmid stabilization/SOS inhibition/maintenance),**  
 571 **yellow (plasmid-encoded fimbriae), white (*Salmonella* plasmid virulence proteins). Outer**  
 572 **circle - black arrows (pSLT-like), red arrows (duplicated region), and white (non-pSLT).**

573 **Figure 2. Comparison of the *spv* genes present on pSLT of *S. Typhimurium* LT2 (upper**  
 574 **panel) and pSTU288-1 (lower panel). The *spvR* and *spvA* genes of *S. Typhimurium* U288**  
 575 **have been deleted following a transposition event that introduced a  $\beta$ -lactamase gene (*bla*<sub>TEM</sub>)**  
 576 **into pSTU288-1. The distal end of the *spv* operon is conserved between the plasmids and**  
 577 **features the remnants of previous transposition event(s).**









578 **Table 1. Plasmid-encoded antibiotic resistance determinants of *S. Typhimurium* U288**

Gene & locus tag	Function	Predicted MW (kDa.)	Pfam domain
<i>sulIII</i> (pSTU288-1_0137)	Dihydropteroate synthase	29	Pterin binding (PF00809) – TIM barrel superfamily (CL0036)
<i>qacEΔ1</i> (pSTU288-1_0139)	Small quaternary ammonium compound resistance	11.8	Small drug resistance (PF00893) – Drug/metabolite transporter superfamily (CL0184)
<i>aadA</i> (pSTU288-1_0140)	Streptomycin 3'-adenylyltransferase/aminoglycoside resistance	29.3	Nucleotidyltransferase_2 (PF01909/CL0260)
<i>cmlA</i> (pSTU288-1_0141)	Chloramphenicol resistance	44.3	Major facilitator superfamily (PF07690/CL0015)
<i>aadA2</i> (pSTU288-1_0142)	Streptomycin 3'-adenylyltransferase/aminoglycoside resistance	29.6	Nucleotidyltransferase_2 (PF01909/CL0260)
<i>dhfr</i> (pSTU288-1_0144)	Dihydrofolate reductase	18.1	DHFR_1 (PF00186) – DHFR (CL0387)
<i>bla</i> <sub>TEM</sub> (pSTU288-1_0177)	Broad-spectrum β-lactamase	31.5	B-lactamase 2 family (PF13354/CL0013)
<i>sulII</i> (pSTU288-2_006)	Dihydropteroate synthase (Type II)	28.5	Pterin binding (PF00809) – TIM barrel superfamily (CL0036)
<i>strA</i> (pSTU288-2_007)	Streptomycin resistance	29.6	Aph phosphotransferase enzyme family (PF01636)
<i>strB</i> (pSTU288-2_008)	Aminoglycoside/hydroxyurea resistance	30.9	Aminoglycoside/hydroxyurea resistance kinase family (PF04655)
<i>tetA</i> (pSTU288-2_013)	Tetracycline resistance	45.2	Major facilitator superfamily (PF07690/CL0015)
<i>cat</i> (pSTU288-2_014)	Chloramphenicol resistance	13.8	Acetyltransferase GNAT family (PF00583/CL0257)

579

580 **Highlights**

581

- 582 • We analyse the plasmids of the emerging pig pathogen *Salmonella* Typhimurium U288
- 583 • Three plasmids encode traits that are of concern for food security and safety
- 584 • Plasmid-encoded genes confer antibiotic resistance and permit genetic mobility
- 585 • The plasmids have a mosaic structure with identifiable horizontal gene acquisitions

586

[Type text]